Influence of Genotype on Susceptibility to Treatment with Fish Oil

Field of the Invention

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The present invention relates to methods for assessing an individual's susceptibility to treatment of an inflammatory disease with a dietary supplement.

10   Background to the Invention

Inflammation in the human body is mediated by tumour necrosis factor-α (TNF-α). TNF-α is one of a group of pro-inflammatory cytokines which appear rapidly following infection and injury

15   (Beutler et al., Crit Care Med **21**:423-35 (1993) and Lin et al., Surgery **127**:117-26 (2000)). TNF-α has widespread effects: it causes loss of lean and adipose tissue, raises body temperature, reduces appetite and stimulates production of a diverse range of immunomodulatory cytokines and oxidant

20   molecules (Grimble, Clin Sci **91**:121-30 (1996)). These effects create a hostile environment for invading pathogens, provide substrate for the immune system from endogenous sources and enhance and modify the activity of the immune system. Thus TNF-α has a pivotal role in allowing the body to withstand

25   pathogenic invasion.

However, excessive or untimely TNF-α plays a major part in mortality and morbidity from sepsis (van der Poll et al., Infect Dis Clin North Am. **13**:413-26 (1999)); meningitis

30   (Westendorp et al., Lancet **349**:170-3 (1997)) and malaria (Knight et al., Proc Assoc Am Physicians **111**:290-8 (1999)). TNF-α also plays an important part in the pathology of inflammatory diseases such as rheumatoid arthritis (Maini et

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al., Ann Rev Med **51**:207-29 (2000)) and inflammatory bowel disease (Murch et al., Gut **32**:913-7 (1991)), in the development of atherosclerotic plaques (Ross, Nature **362**:801-9 1993)) and in rejection of transplanted tissues (Kutukculer et

5   al., Transpl Int. **8**:45-50 (1995)).

Fish oil, which is rich in n-3 polyunsaturated fatty acids (n-3 PUFAs) has been shown to exert an anti-inflammatory influence in a number of animal models of inflammation

10   (Calder, Ann Nutr Metab **41**:203-34 1997) and Grimble, Proc Nutr Soc. **57**:535-42 1998)) and produces anti-inflammatory effects in rheumatoid arthritis (Calder & Zurier Curr Opin Clin Nutr Metab Care **4**:115-21 (2001)), Crohn's disease (Belluzi et al., N Engl J Med **334**:1557-616 (1996)) and psoriasis (Mayser et

15   al., J Am Acad Dermatol **38**:539-47 (1998)). Encapsulated fish oils are often taken as a dietary supplement.

One of the reported anti-inflammatory actions of fish oil is in a reduction in the production of TNF-α by peripheral blood

20   mononuclear cells (PBMC) (Endres et al., N. Engl J Med **320**:265-71 (1989)). However, in studies in which fish oil has been investigated as an anti-inflammatory agent, it has not been found to be effective in all individuals. For example, of eleven studies investigating the effect of fish oil on TNF-α

25   production by PBMC from healthy subjects (Endres et al., N Engl J Med **320**:265-71 (1989); Schmidt et al., Scand J Clin Lab Med **56**:87-92 (1996); Cooper et al., Clin Nutr **12**:321-8 (1993); Blok et al., Eur J Clin Invest **27**:1003-8 (1997); Meydani et al., J Clin Invest **92**:105-13 (1993); Molvig et al., Scan J

30   Immunol **34**:399-410 (1991); Meydani et al., J Nutr **121**:547-55 (1991); Caughey et al., Am J Clin Nutr **63**:116-22 (1996); Yaqoob et al., Eur J Clin Nutr **30**:399-410 (2000); Gallai et al., J Neuroimmunol **56**:143-53 (1995) and Kelley et al., Lipids

34:317-24 (1999)), only six report a suppressive effect
(Endres et al., N Engl J Med **320**:265-71 (1989); Meydani et
al., J Clin Invest **92**:105-13 (1993); Meydani et al., J Nutr
121:547-55 (1991); Caughey et al., Am J Clin Nutr **63**:116-22
5   (1996); Gallai et al., J Neuroimmunol **56**:143-53 (1995) and
Kelley et al., Lipids **34**:317-24 (1999)).

In healthy males and post-menopausal females, TNF-α production
from PBMC shows a remarkable constancy with each individual
10   exhibiting a characteristic level of production of the
cytokine (Jacob et al., PNAS **87**:1233-7 (1990)). However,
among individuals, there is a wide variation in the production
of TNF-α. It is postulated that polymorphisms in the promoter
regions of the TNF-α and lymphotoxin-α (LT-α, also known as
15   TNF-β) genes influence the amount of TNF-α produced following
an inflammatory stimulus (Wilson et al., J Inflamm **45**:1-12
(1995)). This appears to have clinical significance. For
example, individuals homozygous for the TNF-α -308 (TNF2)
allele had 7 times the rate of malaria-related mortality and
20   serious neurological symptoms than individuals who were
heterozygous or homozygous for the more common TNF1 allele (Mc
Guire et al, Nature **371**:508-11 (1994)). Surgical patients
developing sepsis who were homozygous for the LT-α +252
(TNFB2) allele had 3.5 times higher mortality rates than
25   individuals homozygous for the TNFB1 allele, and 2.4 times
higher than individuals who were heterozygous (Stuber et al.,
Crit Care Med **24**:381-4 (1996)).

Levels of a further cytokine, interleukin-6 (IL-6) have also
30   been postulated as determining an individual's response to
fish oil treatment. According to the literature (Fishman et
al., J.Clin Invest **102**:1369-1376 (1998) and Villuendas et al.,
J.Clin Endocrinol Metab **87**:1134-1141 (2002)) individuals with

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a base pair of GG at position -174 of the IL-6 gene produce
raised levels of IL-6, whereas individuals with a CC base pair
at this position produce low levels of IL-6. IL-6 production
in the body contributes to the general level of inflammation
5   experienced by individuals. However, the prior art does not
link the specific genotypes at position -174 with TNF
production. An analysis of the relationship between the
genotype at position -174, the level of inflammation and TNF
production would enable more accurate targeting of fish oil to
10   those individuals most likely to respond thereto.

All of the studies on the effects of fish oil on TNF-α
production reported large standard deviations in TNF-α
production, which could suggest a mixture of genotypes in the
15   study population. It is unknown whether polymorphisms in TNF-
α, LT-α and IL-6 genes influence the ability of fish oil to
suppress TNF-α production. An understanding of these
interactions may explain the inconsistencies in the literature
and may permit more specific targeting of fish oil treatment
20   for inflammatory disease.

Fish oil is an inexpensive treatment for inflammatory disease.
However, rather than suggest to each patient that they should
take the supplement "to see whether it helps" which, if
25   applied to every dietary supplement could result in a large
financial outlay for the patient with no guarantee of success,
it would be preferable to be able to target the fish oil to
those patients most likely to benefit from it.

30   Summary of the Invention

The present inventors have recognised that the sensitivity of
an individual to the inflammation suppressing effects of fish

oil on TNF-α production is linked to genetic variation encoded
by, or associated with, the TNF-α -308, LT-α +252 and IL-6 -
174 single nucleotide polymorphisms (SNP's). The inflammation
suppressing effects of fish oil on TNF-α production have also

5   been shown to be related to the inherent level of production
of TNF-α by cells.

Accordingly, the present invention provides, in its first
aspect, a method of assessing the susceptibility of an

10   individual to treatment of an inflammatory disease with fish
oil, comprising determining the genotype of the individual in
relation to polymorphisms at the TNF-α -308, LT-α +252 and/or
IL-6 -174 alleles; and inferring therefrom whether said
individual responds well to treatment with fish oil.

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In its second aspect, the present invention provides a method
of assessing the susceptibility of an individual to treatment
of an inflammatory disorder with fish oil, comprising:
        a)   determining the inherent TNF-α status of the

20   individual; and
        b)   determining the genotype of the individual in relation
              to polymorphisms at the TNF-α -308, LT-α +252 and/or
              IL-6 -174 alleles; and
inferring therefrom whether said individual responds well to

25   treatment with fish oil.

In a further aspect, the present invention provides a method
for the treatment of an inflammatory disease in a patient,
which comprises assessing the susceptibility of an individual

30   to treatment of an inflammatory disease with fish oil, said
assessment comprising:
        a) determining the genotype of the individual in relation to
           polymorphisms at the TNF-α -308, LT-α +252 and IL-6 -174

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          alleles;

       b) inferring therefrom whether said individual responds well
          to treatment with fish oil; and
       treating said individual with an appropriate amount of fish

5   oil.

       In a yet further aspect, the present invention provides a
       method for the treatment of an inflammatory disease in a
       patient, which comprises assessing the susceptibility of an

10   individual to treatment of an inflammatory disease with fish
       oil, said assessment comprising:
       a)   determining the inherent TNF-α status of the individual;
       b)   determining the genotype of the individual in relation to
       polymorphisms at the TNF-α -308, LT-α +252 and IL-6 -174

15   alleles;
       c)   inferring therefrom whether said individual responds well
       to treatment with fish oil; and
       treating said individual with an appropriate amount of fish
       oil.

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       Detailed Description of the Invention

       In its first aspect, the present invention requires the
       determination of the genotype of the individual in relation to

25   polymorphisms at the TNF-α -308, LT-α +252 and/or IL-6 -174
       alleles.

       Polymorphisms in the TNF-α (TNF1, TNF2) gene and LT-α (TNFB1,
       TNFB2) gene will result in three genotypes in each case,

30   homozygous TNF1/1 or TNFB1/1, or homozygous TNF2/2 and TNFB2/2
       or heterozygous TNF1/2 and TNFB1/2. In a similar fashion, in
       respect of the IL-6 gene, individuals will either be
       homozygous CC or GG or heterozygous CG. Thus, in the present

invention an individual may have one of the following
genotypes:

homozygous TNF1/1 : homozygous TNFB1/1 : homozygous IL-6 CC
5 homozygous TNF1/1 : homozygous TNFB1/1 : homozygous IL-6 GG
homozygous TNF1/1 : homozygous TNFB1/1 : heterozygous IL-6 CG
homozygous TNF1/1 : homozygous TNFB2/2 : homozygous IL-6 CC
homozygous TNF1/1 : homozygous TNFB2/2 : homozygous IL-6 GG
homozygous TNF1/1 : homozygous TNFB2/2 : heterozygous IL-6 CG
10 homozygous TNF1/1 : heterozygous TNFB1/2 : homozygous IL-6 CC
homozygous TNF1/1 : heterozygous TNFB1/2 : homozygous IL-6 GG
homozygous TNF1/1 : heterozygous TNFB1/2 : heterozygous IL-6 CG
homozygous TNF2/2 : homozygous TNFB1/1 : homozygous IL-6 CC
homozygous TNF2/2 : homozygous TNFB1/1 : homozygous IL-6 GG
15 homozygous TNF2/2 : homozygous TNFB1/1 : heterozygous IL-6 CG
homozygous TNF2/2 : homozygous TNFB2/2 : homozygous IL-6 CC
homozygous TNF2/2 : homozygous TNFB2/2 : homozygous IL-6 GG
homozygous TNF2/2 : homozygous TNFB2/2 : heterozygous IL-6 CG
homozygous TNF2/2 : heterozygous TNFB1/2 : homozygous IL-6 CC
20 homozygous TNF2/2 : heterozygous TNFB1/2 : homozygous IL-6 GG
homozygous TNF2/2 : heterozygous TNFB1/2 : heterozygous IL-6 CG
heterozygous TNF1/2 : homozygous TNFB1/1 : homozygous IL-6 CC
heterozygous TNF1/2 : homozygous TNFB1/1 : homozygous IL-6 GG
heterozygous TNF1/2 : homozygous TNFB1/1 : heterozygous IL-6 CG
25 heterozygous TNF1/2 : homozygous TNFB2/2 : homozygous IL-6 CC
heterozygous TNF1/2 : homozygous TNFB2/2 : homozygous IL-6 GG
heterozygous TNF1/2 : homozygous TNFB2/2 : heterozygous IL-6 CG
heterozygous TNF1/2 : heterozygous TNFB1/2 : homozygous IL-6 CC
heterozygous TNF1/2 : heterozygous TNFB1/2 : homozygous IL-6 GG; or
30 heterozygous TNF1/2 : heterozygous TNFB1/2 : heterozygous IL-6 CG.

Although on paper the above are all of the possible gene
combinations, in reality, not all of the combinations will
occur. Certain combinations may be prevented from forming,
35 for example due to the linkage of some genes, causing linkage

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disequilibrium.   In addition to this, the gene TNF2/2 is very
rare, occurring in less than 10% of the population.

5   In a preferred embodiment of the present invention, it is
preferred that the genotype at the LT-α gene is heterozygous
TNFB1/2.

In a further preferred embodiment of the present invention, it
is preferred that the genotype at the IL-6 gene is homozygous
10   GG.

In a more preferred embodiment of the present invention, it is
preferred that the genotype at the LT-α and IL-6 genes is
heterozygous TNFB1/2 and IL-6 GG respectively.
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In a preferred embodiment of this aspect of the present
invention, the genotype at one of the TNF-α, LT-α and IL-6
alleles is determined.   It is more preferred however, that the
genotype of two or more of the TNF-α, LT-α and IL-6 alleles is
20   determined and most preferred that the genotype of both the
LT-α and IL-6 alleles is determined.

In its second aspect, the present invention requires the
determination of the inherent TNF-α status of an individual.
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The inherent TNF-α status of an individual is a measurement of
the ability of that individual's white blood cells to make
TNF.   The inherent TNF-α status of an individual in a disease-
free or substantially disease-free state demonstrates a
30   remarkable constancy.   The production of TNF-α is generally
not affected by age or sex of the individual.   By disease-free
or substantially disease-free is meant that the individual
does not suffer from any type or significant level of

inflammatory disorder. The inherent TNF-α status of an
individual is thus preferably determined when the individual
is not suffering from any type or significant level of
inflammatory disorder.

Determination of the inherent TNF-α status of an individual
may be made using procedures known to the person skilled in
the art. Typically samples of whole blood are collected and
peripheral blood mononuclear cells (PBMC) isolated therefrom.
Techniques for isolation of PBMC are known and include, for
example, treatment of whole blood with lithium heparin,
followed by centrifugation to isolate PBMC, after which TNF-α
concentrations can be measured, for example, using standard
methods such as EASIA® ELISA kits (Biosource International,
Nivelles, Belgium).

TNF-α producers can be divided into numerous groups depending
on the concentration of TNF-α produced by their PBMC and
thereby defining their inherent TNF-α status. It is generally
preferred for the purposes of the present invention that
producers be classified into three groups, for example as
high, medium or low producers. Although the exact amounts of
TNF-α produced by the PBMC are not essential to define the
above three groups, it is generally preferred that a high
producer has a concentration of TNF-α of about 850 - 2500 ng/L
of incubate from $1x10^9$ cells, a medium producer has a TNF-α
concentration of 2500 - 5000 ng/L of incubate from $1x10^9$ cells
and a high producer has a concentration of TNF-α of about 5000
- 14000 ng/L of incubate from $1x10^9$ cells.
Table 1, below, illustrates the effects of fish oil on TNF-α
production.

Table 1

| Tertile | No. subjects | Conc. TNF-α – ng/L | |
		Pre-Supplementation	Post-supplementation
	111	4821±4177	4643±3338
Low	37	1458±600	3809±2571*
Medium	37	3728±936	4796±3270
High	37	9277±4338	5323±3941*

*significantly different from pre-supplementation value

5   ($P<0.05$; Student's paired t-test)

Differences between pre- and post-fish oil supplementation
values for TNF-α production were determined using Student's
paired t-test. It is apparent from the above results that

10   sensitivity to fish oil administration is influenced by pre-
supplementation or inherent TNF-α production. In the highest
tertile, mean TNF-α production was reduced by 43%. TNF-α
production was reduced in the middle tertile, although not by
a significant amount and in the lowest tertile, TNF-α

15   production was increased by 160%.

In view of the above results, the present inventors have
determined that an individual whose inherent TNF-α production
causes them to fall into the "low producer" category, i.e. an

20   individual having from about 850 – 2500 ng TNF-α/L of incubate
from $1x10^9$ cells, is not likely to respond well to fish oil
treatment. In contrast, an individual whose inherent TNF-α
production causes them to fall into the "high producer"
category, i.e. an individual having from about 5000 – 14000 ng

TNF-α/ L of incubate from 1×10^9 cells is likely to respond well
to fish oil treatment.

A genomic sample suitable for use in such a method may be
isolated from any suitable client or patient cell sample. For
convenience, it is preferred that the DNA is isolated from
cheek (buccal) cells. This enables easy and painless
collection of cells.

Cells may be isolated from the inside of the mouth using a
disposable scraping device with a plastic or paper matrix
"brush", for example, the C.E.P. Swab™ (Life Technologies
Ltd., UK). Cells are deposited onto the matrix upon gentle
abrasion of the inner cheek, resulting in the collection of
approximately 2000 cells (Aron, Y. et al (1994) Allergy 49
(9): 788-90). The paper brush can then be left to dry
completely, ejected from the handle placed into a micro-
centrifuge tube for storage prior to analysis.

Genomic DNA from the cell samples may be isolated using
conventional procedures. For example DNA may be immobilised
onto filters, column matrices, or magnetic beads. Numerous
commercial kits, such as the Qiagen QIAamp kit (Qiagen,
Crawley, UK) may be used. Briefly, the cell sample may be
placed in a microcentrifuge tube and combined with Proteinase
K, mixed, and allowed to incubate to lyse the cells. Ethanol
is then added and the lysate is transferred to a QIAamp spin
column from which DNA is eluted after several washings.

The amount of DNA isolated by the particular method used may
be quantified to ensure that sufficient DNA is available for
the assay and to determine the dilution required to achieve
the desired concentration of DNA for PCR amplification. For

example, the desired target DNA concentration may be in the range 50 ng and 150 ng. DNA concentrations outside this range may impact the PCR amplification of the individual alleles and thus impact the sensitivity and selectivity of the

5   polymorphism determination step.

The quantity of DNA obtained from a sample may be determined using any suitable technique. Such techniques are well known to persons skilled in the art and include UV (Maniatis T.,

10   Fritsch E. F., and Sambrook J., (1982) Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Springs Harbor, NY) or fluorescence based methods. As UV methods may suffer from the interfering absorbance caused by contaminating molecules such as nucleotides, RNA, EDTA and phenol and the

15   dynamic range and sensitivity of this technique is not as great as that of fluorescent methods, fluorescence methods are preferred. Commercially available fluorescence based kits such as the PicoGreen dsDNA Quantification (Molecular Probes, Eugene, Oregon, USA).

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Prior to the testing of a sample, the nucleic acids in the sample may be selectively amplified, for example using Polymerase Chain Reaction (PCR) amplification, as described in U.S. patent numbers 4,683,202 and 4,683,195.

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Preferred primers for use in the present invention are from 18 to 23 nucleotides in length, without internal homology o primer-primer homology.

30   Furthermore, to ensure amplification of the region of interest and specificity, ARMS-PCR or allele specific PCR methodology is used. In this way, in each PCR reaction, the 3' end of one primer is located exactly at the site of the SNP, so that

amplification can only occur if the nucleotide base
corresponding to one particular allele is present. Tow PCR
reactions are performed per SNP, one specific for each allele.
One primer is common to both PCR reactions, while separate
5   allele specific primers are added for each reaction, e.g. the
common primer could be "forward" with allele 1 (reaction 1) or
allele 2 (reaction 2) added as "reverse" primers, or vice-
versa.

10   This approach is based upon the original discovery that
specificity in a PCR reaction is dependent upon precise matching
of the terminal 3' end of a PCR primer and its target DNA
sequence. Using this approach, point mutations may be
distinguished from wild-type sequences using a single generic
15   primer combined with one of two antisense primers in separate
PCR reactions. One of the antisense primers is precisely
matched with the wild-type sequence at its 3' end, while the
second primer is precisely matched with the mutant sequence at
its 3' end. Therefore one PCR reaction will only amplify the
20   wild-type sequence and the other will only amplify the mutant
sequence. This method, originally termed the 'amplification
refractory mutation system-PCR' (ARMS-PCR) (Newton et al 1989),
is dependent upon the fact that Taq DNA polymerase lacks 3' to
5' exonucleolytic proof-reading activity, so that Watson-Crick
25   mismatches at the 3' end of the primer-template duplex cannot be
corrected, which would result in mispriming. Successful
application of the ARMS-PCR approach also requires stringent
conditions for primer annealing in the PCR reaction. A vital
component of this methodology is inclusion of a second primer
30   pair which amplifies a sequence from a second gene, to act as an
in-tube positive control for successful or failed PCR
amplification in that tube.

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Preferred examples of primer pairs which may be used for
analysing the TNF-α, LT-α and IL-6 genes are shown in Table 2,
together with the control primers which amplify a sequence
from the third intron of the human leukocyte antigen DRB1, to
5 act as an internal control for successful PCR.

Table 2

SNP	Primer Name	Primer Sequence (5'-3')	Seq ID	Prod. Size (BPs)
TNF-α -308	TNF308 common	TCTCGGTTTCTTCTCCATCG	1	184
	TNF308G	ATAGGTTTTGAGGGGCATGG	2	
	TNF308A	ATAGGTTTTGAGGGGCATGA	3	
LT-α +252	LT252 common	AGATCGACAGAGAAGGGGACA	4	94
	LT252G	CATTCTCTGTTTCTGCCATGG	5	
	LT252A	CATTCTCTGTTTCTGCCATGA	6	
IL-6 -174	IL6174 common	TTTGTTGGAGGGTGAGGGTGG	7	108
	IL6174G	TTCCCCCTAGTTGTGTCTTGCG	8	
	IL6174C	TTCCCCCTAGTTGTGTCTTGCC	9	
Control primers	63	TGCCAAGTGGAGCACCCAA	10	796
	64	GCATCTTGCTCTGTGCAGAT	11	

10 Having obtained a sample of DNA, preferably with amplified
regions of interest, the individual polymorphisms may be
identified. Identification of the markers for the
polymorphisms involves the discriminative detection of allelic
forms of the TNF-α, LT-α and IL-6 genes that differ by
15 nucleotide substitution at positions -308, +252 and/or -174
respectively.

Methods for determining the presence of known nucleotide

differences are well known to the skilled person. These may
include, but are not limited to:

- Hybridization with allele-specific oligonucleotides (ASO),
5   (Wallace, R. B. et al (1981) Nucleic Acids Research. 9:879-
894; Ikuta, S. et al (1987) Nucleic Acids Research. 15:797-
811; Nickerson, D. et al(1990) PNAS USA 87:8923-8927, Verlaan-
de Vries, M et al (1986) Gene. 50:313-320, Saiki, R. K. et al
(1989) PNAS. USA 86:6230-6234 and Zhang, Y. et al (1991)
10   Nucleic Acids Research. 19: 3929-3933)

- Allele specific PCR, (Newton, C. R. et al (1989). Nucleic
Acids Research. 17:2503-2516, Gibbs, R. A. et al (1989)
Nucleic Acids Research. 17:2437-2448).
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The following reference gives full details of the TNF alpha/LT
alpha genotyping system used:

- Howell WM, Bateman AC, Turner SJ, Theaker JM (2002). Influence
20   of TNFα and LTα single nucleotide polymorphisms on
susceptibility to and prognosis in cutaneous malignant melanoma
in the British population European Journal of Immunogenetics,
29, 17-23.

25   A few of other examples describing details of cytokine
genotyping by ARMS-PCR include:

- McCarron SL, Edwards S, Evans PR, Gibbs R, Dearnaley DP, Dowe
A, Southgate C, The CRC/BPG UK Familial Prostate Cancer Study
30   Collaborators, Easton DF, Eeles RA, Howell WM (2002) Influence
of cytokine gene polymorphisms on the development of prostate
cancer. Cancer Research, 62, 3369-3372.

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- Howell WM, Turner SJ, Bateman AC, Theaker JM (2001). IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. Genes and Immunity, 2, 25-31.

5   - Poole KL, Gibbs PJ, Evans PR, Sadek SA, Howell WM (2001) Influence of patient and donor cytokine genotypes on renal allograft rejection: evidence from a single study. Transplant Immunology, 8, 259-265.

10   Further references of interest include:

- Solid-phase minisequencing (Syvanen, A. C. et al(1993) Am. J. Human Genet. 52:46-59).

15   - Oligonucleotide ligation assay (OLA) (Wu, D. Y., et al (1989) Genomics. 4:560-569, Barany, F. (1991) PNAS USA 88:189-193, Abravaya, K. et al 1995. Nucleic Acids Research. 23:675-682).

20   - The 5' fluorogenic nuclease assay (Lee, E. et al J. Toxicol. Soc. 23: 140-142, (1998), US4,683,202, US4,683,195, US5,723,591 and US5,801,155).

- Restriction fragment length polymorphism (RFLP), (Donis-
25   Keller H. et. al. (1987) Cell, 51, 319-337).

In a preferred embodiment, the loci for the three genes may be assessed via a specialised type of PCR used to detect polymorphisms, commonly referred to as the Taqman® assay and
30   performed using a AB7700 OR 7900HT instruments (Applied Biosystems, Warrington, UK). In this method, a probe is synthesised which hybridises to a region of interest containing the polymorphism. The probe contains three

modifications: a fluorescent reporter molecule, a fluorescent
quencher molecule and a minor groove binding chemical to
enhance binding to the genomic DNA strand. The probe may be
bound to either strand of DNA. For example, in the case of

5   binding to the coding strand, when the Taq polymerase enzyme
begins to synthesise DNA from the 5' upstream primer, the
polymerase will encounter the probe and begin to remove bases
from the probe one at a time using a 5'-3' exonuclease
activity. When the base bound to the fluorescent reporter

10   molecule is removed, the fluorescent molecule is no longer
quenched by the quencher molecule and the molecule will begin
to fluoresce. This type of reaction can only take place if
the probe has hybridised perfectly to the matched genomic
sequence. As successive cycles of amplification take place,

15   i.e. more probes and primers are bound to the DNA present in
the reaction mixture, the amount of fluorescence will increase
and a positive result will be detected. If the genomic DNA
does not have a sequence that matches the probe perfectly, no
fluorescent signal is detected.

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The results of the genetic polymorphism analysis may be used
in combination with the results of the determination of the
producer level with respect to inherent TNF-α production to
allow a determination to be made of the susceptibility of the

25   individual to fish oil treatment. Table 3, below,
demonstrates the distribution of TNF-α, LT-α and IL-6
genotypes in the study population in relation to inherent TNF-
α producer status.

Table 3

Tertile	TNF-α genotype			LT-α Genotype(B)			IL-6 genotype		
	1/1	1/2	2/2	1/1	1/2	2/2	GG	GC	CC
All	76	33	2	21	59	31	49	45	17
Low	25	11	1	8	23	6	16	11	10
Medium	25	11	1	8	19	10	14	18	5
High	26	11	0	5	17	15*	19	16	2

*indicates distribution of LT-α and IL-6 genotypes

5   significantly different from lowest tertile of TNF-α

production ($P<0.001$:χ^2 test).

As may be seen from the above table, the percentage of
subjects falling into the TNF1/1, TNF1/2 and TNF2/2 genotypes

10   was approximately 68%, 30% and 2% respectively. The
percentage of subjects falling into the TNFB1/1, TNFB1/2 and
TNFB2/2 genotypes was 19%, 53% and 28% respectively and the
percentage of subjects falling into the IL-6GG, IL-6GC and IL-
6CC genotypes was 53%, 56% and 29% respectively. TNF-α

15   genotype appeared to be unrelated to TNF-α production since
the distribution of TNF1 and TNF2 alleles was almost identical
for the subjects in all tertiles of pre-supplementation TNF-α
production. The frequency of the TNFB2/B2 and IL-6 GG
genotypes was related positively to TNF-α production,

20   increasing in the case of TNFB2/2 from 19% in the lowest
tertile to 48% in the highest tertile and in the case of IL-6
showing a smaller increase of from 33% in the lowest tertile
to 39% in the highest. The frequency of the TNFB1/B2 genotype

and the IL-6 CC genotype declined as inherent TNF-α production increased.

The influence of genotype on the ability of fish oil to reduce
5   TNF-α production from LPS stimulated peripheral blood mononuclear cells is also demonstrated in Table 4. It is apparent from this table that the presence of either the IL-6 GG or TNFB 1/2 polymorhisms alone causes the individual with either of those genotypes to be more susceptible to the
10   beneficial lowering of TNF-α production following fish oil administration. Furthermore, a combination of the TNFB1/2 and IL-6GG genotypes is most likely to result in enhancing the effects of fish oils reduction of TNF production, as is shown by the fact that 56% of subjects with that genotype show a
15   reduction in TNF-α production following fish oil administration (Table 4).

Table 4
Influence of genotype on the ability of fish oil (6g/d for 12 weeks) to reduce TNF-alpha production from LPS stimulated peripheral blood mononuclear cells of men.

Genotype combinations	Total subjects	Number showing a fall in TNF after fish oil	Number showing no change or a rise in TNF after fish oil	Percentage of subjects with genotype showing a fall in TNF after fish oil
TNFB11 & IL-6 GG	12	4	8	33
TNFB12 & IL-6 GG	45	25	20	56
TNFB22 & IL6 GG	32	15	16	47
TNFB11 & IL-6 GC	12	4	8	33
TNFB12 & IL-6 GC	41	19	22	46
TNFB22 & IL-6 GC	34	15	19	44
TNFB11 & IL-6 CC	5	1	4	20
TNFB12 & IL-6 CC	18	6	12	33
TNFB22 & IL-6 CC	9	1	8	11
TNFB11	29	9	20	31
TNFB12	104	51	53	49
TNFB22	75	31	44	41
IL-6 GG	89	44	45	49
IL-6 GC	87	38	49	44
IL-6 CC	32	8	24	25
Irrespective of genotype	208	90	118	43

A subject is deemed to have shown a fall ion TNF-alpha production after fish oil if post-fish oil value is >10% lower than pre-fish oil value.
TNF-beta +252 and IL-6 −174 Single nucleotide polymorphisms characterized.
10-10-2002

21

For the purposes of the present invention, fish oil is oil
extracted from the flesh and organs of fish which contains at
least 28% n-3 PUFAs, of which about 60% is eicosapentaenoic
acid and about 40% is docosahexaenoic acid. The fish oil may
5   be extracted from any oily fish source. Particularly
appropriate in this respect are mackerel, sprats, herring,
tuna and wild salmon, these fish being rich sources of n-3
PUFAs. The remaining components of the fish oil are typically
a mixture of saturated and monounsaturated fatty acids which
10   do not appear to have any bearing on the activity of the fish
oil.

A suitable inflammatory disorder may include any disorder in
which a reduction in inflammation is desirable, including an
15   inflammatory skin disorder such as atopic dermatitis, contact
dermatitis, eczema, psoriasis and other inflammatory disorders
such as Perianal Crohn's disease and arthritis, for example,
rheumatoid or psoriatic arthritis. Fish oil treatment has
been found to be of particular benefit in the treatment of
20   rheumatoid arthritis.

In some embodiments, a method may comprise the further step of
administering fish oil to the individual. Fish oil may be
administered alone or in combination with one or more
25   ofvitamin B12/B6 and antioxidants, for example vitamin C,
vitamin E, lycopene, beta-carotene and minerals such as
magnesium, manganese, selenium and zinc.

Administration may be in the form of a medicament such as a
30   tablet or pill, which, for example comprises the active
ingredient and a suitable excipient, or in the form of a
foodstuff rich in fish oil. Suitable foodstuffs would include
oily fish such as mackerel, sprats, herring, tuna and wild

22

salmon.

A method may comprise the further step of providing a dietary
regime for said individual comprising foodstuffs comprising
5   elevated levels of one or more of folic acid, vitamin B6/B12
and vitamin C.

Another aspect of the invention provides the use of
composition comprising fish oil in the manufacture of a
10   medicament for use in the treatment of an inflammatory
disorder in an individual who is polymorphic for one or more
of the TNF-α -308, LT-α $+252$ and IL-6 -174 polymorphisms.

The term "treatment" as used herein in the context of treating
15   a condition, pertains generally to treatment and therapy,
whether of a human or an animal (e.g. in veterinary
applications), in which some desired therapeutic effect is
achieved, for example, the inhibition of the progress of the
inflammatory condition, and includes a reduction in the rate
20   of progress, a halt in the rate of progress, amelioration of
the inflammatory condition, and cure of the inflammatory
condition. Treatment as a prophylactic measure (i.e.
prophylaxis) is also included.

25   The term "therapeutically-effective amount" as used herein,
pertains to that amount of an active compound, or a material,
composition or dosage from comprising an active compound,
which is effective for producing some desired therapeutic
effect, commensurate with a reasonable benefit/risk ratio.

30

Fish oil or a pharmaceutical composition comprising fish oil,
may be administered to a subject by any convenient route of
administration, including but not limited to oral

administration (e.g. by ingestion) or parenteral
administration, e.g. by subcutaneous, intramuscular or
intravenous injection.

5   The subject may be a eukaryote, an animal, a vertebrate
     animal, a mammal, a rodent, murine, canine, feline, equine
     bovine, ovine or human.

     While it is possible for the fish oil to be administered
10   alone, it is preferable to present it as a pharmaceutical
     composition (e.g. formulation) comprising at least the fish
     oil together with one or more pharmaceutically acceptable
     carriers, adjuvants, excipients, diluents, fillers, buffers,
     stabilisers, preservatives, lubricants, or other materials
15   well known to those skilled in the art and optionally other
     therapeutic or prophylactic agents.

     Thus, the present invention further provides pharmaceutical
     compositions, as defined above, and methods of making a
20   pharmaceutical composition comprising admixing fish oil
     together with one or more pharmaceutically acceptable
     carriers, excipients, buffers, adjuvants, stabilisers, or
     other materials, as described herein.

25   The term "pharmaceutically acceptable" as used herein pertains
     to compounds, materials, compositions, and/or dosage forms
     which are, within the scope of sound medical judgement,
     suitable for use in contact with the tissues of a subject
     (e.g. human) without excessive toxicity, irritation, allergic
30   response, or other problem or complication, commensurate with
     a reasonable benefit/risk ratio. Each carrier, excipient,
     etc. must also be "acceptable" in the sense of being
     compatible with the other ingredients of the formulation.

24

Suitable carriers, excipients, etc. can be found in standard
pharmaceutical texts, for example, Remington's Pharmaceutical
Sciences, 18th edition, Mack Publishing Company, Easton, Pa.,
1990.

5

The formulations may conveniently be presented in unit dosage
form and may be prepared by any methods well known in the art
of pharmacy. Such methods include the step of bringing into
association the active compound with the carrier which

10   constitutes one or more accessory ingredients. In general,
the formulations are prepared by uniformly and intimately
bringing into association the active compound with liquid
carriers or finely divided solid carriers or both, and then if
necessary shaping the product.

15

Formulations may be in the form of liquids, solutions,
suspensions, emulsions, elixirs, syrups, tablets, lozenges,
granules, powders, capsules, cachets, pills, ampoules, oils,
suppositories, boluses or sustained release formulations.

20

Formulations suitable for oral administration (e.g. by
ingestion) may be presented as discrete units such as
capsules, cachets or tablets, each containing a predetermined
amount of the active compound; as a powder or granules; as a

25   solution or suspension in an aqueous or non-aqueous liquid; or
as an oil-in-water liquid emulsion or a water-in-oil liquid
emulsion; as a bolus; as an electuary; or as a paste.

A tablet may be made by conventional means, e.g., compression

30   or moulding, optionally with one or more accessory
ingredients. Compressed tablets may be prepared by
compressing in a suitable machine the active compound in a
free-flowing form such as a powder or granules, optionally

mixed with one or more binders (e.g. povidone, gelatin, acacia, sorbitol, tragacanth, hydroxypropylmethyl cellulose); fillers or diluents (e.g. lactose, microcrystalline cellulose, calcium hydrogen phosphate); lubricants (e.g. magnesium
5   stearate, talc, silica); disintegrants (e.g. sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose); surface-active or dispersing or wetting agents (e.g. sodium lauryl sulfate); and preservatives (e.g. methyl p-hydroxybenzoate, propyl p-hydroxybenzoate,
10 sorbic acid). Moulded tablets may be made by moulding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active compound therein using,
15 for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

20 Parenteral administration is generally characterized by injection, either subcutaneously, intramuscularly or intravenously. Injectables can be prepared in conventional forms, either as liquid solutions or suspensions, solid forms suitable for solution or suspension in liquid prior to
25 injection, or as emulsions. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like. In addition, if desired, the pharmaceutical compositions to be administered may also contain minor amounts of non-toxic auxiliary substances such as wetting or
30 emulsifying agents, pH buffering agents and the like, such as for example, sodium acetate, sorbitan monolaurate, triethanolamine oleate, triethanolamine sodium acetate, etc.

26

A more recently devised approach for parenteral administration
employs the implantation of a slow-release or sustained-
release system, such that a constant level of dosage is
maintained. See, e.g., US Patent No. 3,710,795.

5

The percentage of active compound contained in such parental
compositions is highly dependent on the specific nature
thereof, as well as the activity of the compound and the needs
of the subject. However, percentages of active ingredient of

10   0.1% to 10% in solution are employable, and will be higher if
the composition is a solid which will be subsequently diluted
to the above percentages. Preferably, the composition will
comprise 0.2-2% of the active agent in solution.

15   It will be appreciated that appropriate dosages of the active
compounds, and compositions comprising the active compounds,
can vary from patient to patient. Determining the optimal
dosage will generally involve the balancing of the level of
therapeutic benefit against any risk or deleterious side

20   effects of the treatments of the present invention. The
selected dosage level will depend on a variety of factors
including, but not limited to, the activity of the particular
compound, the route of administration, the time of
administration, the rate of excretion of the compound, the

25   duration of the treatment, other drugs, compounds, and/or
materials used in combination, and the age, sex, weight,
condition, general health, and prior medical history of the
patient. The amount of compound and route of administration
will ultimately be at the discretion of the physician,

30   although generally the dosage will be to achieve local
concentrations at the site of action which achieve the desired
effect without causing substantial harmful or deleterious
side-effects.

Administration *in vivo* can be effected in one dose,
continuously or intermittently (e.g. in divided doses at
appropriate intervals) throughout the course of treatment.
Methods of determining the most effective means and dosage of
5   administration are well known to those of skill in the art and
will vary with the formulation used for therapy, the purpose
of the therapy, the target cell being treated, and the subject
being treated. Single or multiple administrations can be
carried out with the dose level and pattern being selected by
10   the treating physician.

In general, a suitable dose of fish oil is in the range of
about 4g to about 8g per day, more preferably about 6g per
day. This may be in the form of a single bolus dose or more
15   preferably in multiple applications or a sustained release
preparation. Factors such as age, weight, sex and presence or
absence of other, non-inflammatory, diseases, will generally
not have a bearing on the suitable daily dose of fish oil.

20   The precise format for performing methods of the invention may
be varied by those of skill in the art using routine skill and
knowledge.

Of course, the person skilled in the art will design any
25   appropriate control experiments with which to compare results
obtained in methods of the invention.

Aspects of the present invention will now be illustrated with
reference to the experiments and results below, by way of
30   example and not limitation. Further aspects and embodiments
will be apparent to those of ordinary skill in the art.

All documents mentioned in this specification are hereby

incorporated herein by reference.

Experiments

5   1.   Subjects and study design

Healthy male subjects ($n = 111$), age 28 ± 8 years (range 20-57
years), body weight 77 ± 11 kg (range 50-103 kg) and body mass
index 24 ± 4 kg/m^2 (range 18-34 kg/m^2), were recruited from the
10 Southampton area. Smokers and individuals with inflammatory
disease or on anti-inflammatory drugs were excluded from the
study. Subjects continued with their normal lifestyles and
diet but, in addition, consumed 6 g/d of encapsulated fish oil
(providing 1.8 ng n-3 PUFAs/d) (Maxepa, Seven Seas Ltd, Hull,
15 UK) for 12 weeks. Prior to providing blood subjects fasted
overnight, for at least 12 hours. Three separate blood
samples were taken sequentially at the start and on completion
of the period of fish oil supplementation. First 20 ml of
blood was taken into a vacutainer tube containing lithium
20 heparin; this blood was used for preparation of PBMC. Then 5
ml of blood was taken into a coagulant-free vacutainer tube:
this blood was used for preparation of serum to measure C-
reactive protein (CRP) concentrations. Finally 5 ml of blood
was taken into a vacutainer containing EDTA: this blood was
25 used to prepare DNA for genotyping.

Serum CRP concentrations were measured in order to detect the
presence of infection or inflammation in the subjects at the
time of blood sampling. Individuals with CRP concentrations >
30 100 mg/L from either blood sample were excluded from the
study.

29

2.   TNF-α induction and measurement

PBMC were isolated by centrifugation of heparinised blood on
Histpaque-1077 (Sigma Chemical Co., Poole, UK) (Yaqoob et al,.
5   Eur J Clin Nutr **30**:399-410 (2000)) and were resuspended in
RPMI culture medium containing 2 mmol/L glutamine and 50 ml/l
autologous plasma. PBMC (2 x 10^6) were cultured in 24 well
tissue culture plates in the presence of a final concentration
of 15 mg/l *Eschericia coli* 0111:B4 endotoxin (Sigma Chemical
10 Co., Poole, UK) in a final culture volume of 2 ml. After 24h
at 37°C in a 5% CO_2/95% air atmosphere, the culture plates were
centrifuged and the supernatants frozen at -80°C until
analysis. TNF-α concentrations were measured using EASIA®
ELISA kits (Biosource International, Nivelles, Belgium). The
15 inter- and intra-assay coefficients of variation were <10% and
the limit of detection was 3ng/l.

3.   Genotyping for TNF-α and LT-α alleles

20 An aliquot of blood which had been collected into EDTA was
genotyped for the TNF-α -308 (TNF1, TNF2) and LT-α +252
(TNFB1, TNFB2) single nucleotide polymorphisms (SNP's). These
SNPs were selected due to their documented, but variable,
association with TNF-α production (Kroeger et al., Cytokine
25 **12**:110-119 (2000) and Warzocha et al., Blood **91**:3564-81
(1998)). Genomic DNA was extracted by a salting out procedure
(Miller et al., Nucleic Acid Res **16**:1215 (1988)). Each SNP
was detected using a two reaction amplification refractory
mutation system polymerase chain reaction (ARMS-PCR) approach
30 based on previously published methods (Howell WM et al.,
European Journal of Immunogenetics, **29**:17-23 (2002); Perrey et
al., Transpl Immunol **7**:127-8 (1999)). In this approach, two
separate PCR reactions per SNP are performed. Each PCR

reaction also contained an additional pair of PCR primers,
amplifying a sequence from the third intron of the human
leucocyte antigen DRB1 gene to act as an internal control for
successful PCR. All PCR reactions were performed in 10μl

5   reaction volumes and final reagent concentrations were as
follows: 1 x AS reaction buffer (Abgene, Epsom, UK), 200
μmol/l each dNTP, 120 g/l sucrose, 200 μmol/l cresol red, 1
μmol/l each specific or common primer, 0.2 μmol/l each
internal control primer, 0.25 units Thermoprime[PLUS] DNA

10 polymerase (Abgene, Epsom, UK), 1.75 mmol/l $MgCl_2$ and 25-100ng
DNA. PCR primer sequences and product sizes for each SNP
amplicon are given in Table 2. PCR reaction conditions were
performed using a Primus 96 Plus thermal cycler (MWG Biotech,
Germany) according to the following cycling conditions: 96°

15 for 60s, followed by ten cycles of 96° for 15s, 65° for 50s,
72° for 40s; then twenty cycles of 96° for 190s, 60° for 50s,
72° for 40s. PCR products were loaded directly onto 2%
agarose gels containing 0.5 g/l ethidium bromide,
electrophoresed and visualised by photography under UV

20 transillumination.

4. Plasma phospholipid fatty acid composition

Compliance to the dietary fish oil treatment was assessed by

25 determination of the fatty acid composition of plasma
phospholipids. Total lipid was extracted from plasma with
chloroform/methanol (2:1 v/v) and phospholipids were isolated
by thin layer chromatography using a mixture of hexane/diethyl
ether/acetic acid (90:30:1 v/v/v) as the elution phase. Fatty

30 acid methyl esters were prepared by incubation with 10 g/l
boron trifluoride in methanol at 80°C for 60 mins. Fatty acid
methyl esters were isolated by solvent extraction, dried and
separated by gas chromatography in a Hewlett-Packard 6890 gas

chromatograph (Hewlett Packard, Avondale, PA) fitted with a
30m x 0.32mm BPX70 capillary column, film thickness 0.25μm.
Helium at 1.0 ml/min was used as the carrier gas and the
split/splitless injector was used with a split/splitless ratio

5   of 20:1. Injector and detector temperatures were 275°C. The
column oven temperature was maintained at 170°C for 12 min
after sample injection and was programmed then to increase
from 170 to 210°C at 5°C/min before being maintained at 210°C
for 15 min. The separation was recorded with HP GC Chem

10 Station software (Hewlett Packard, Avondale, PA). Fatty acid
methyl esters were identified by comparison with standards run
previously.

5.   Statistical Analysis

15

Unless otherwise indicated values are expressed as mean ± SD.
Differences in the distribution of TNF-α and LT-α genotypes
among the tertiles of TNF-α production before fish oil
supplementation were examined using the χ^2 test. Differences

20 between the pre- and post-fish oil supplementation values for
TNF-α production and for the proportions of various fatty
acids in plasma phospholipids were determined using Students
paired t-test. Differences in TNF-α production among
different genotypes, either before or after fish oil

25 supplementation, were determined by one-factor ANOVA. The
influence of genotype, of tertile of pre-supplementation TNF-α
production and of their interaction on the effect of fish oil
on TNF-α production were determined by two-factor ANOVA. In
all cases, the level of significance was set at 0.05 and

30 Bonferonni's correction for multiple comparisons was used.
All statistical comparisons were made using SPSS Version 10
(SPSS Inc, Chicago, IL).

Results

1.　　Plasma phospholipid fatty acid composition

All subjects showed an increase in the proportions of
eicosapentaenoic acid and docosahexaenoic acid in their plasma
phospholipids, with mean increases of 370 and 94 respectively,
at the end of the supplementation period (Table 5). Increased
appearance of the fish oil-derived n-3 PUFAs was accompanied
by a significant decrease in the proportion of arachidonic
acid in plasma phospholipids.

Table 5

Fatty Acid	% by wt of total fatty acids	
	Pre-supplementation	Post-supplementation
Eicosapenaenoic acid (20:5n-3)	0.72 ± 0.09	3.34 ± 0.29*
Docosahexaenoic acid (22:6n-3)	1.97 ± 0.27	3.68 ± 0.27*
Arachidonic acid (20:4n-6)	7.66 ± 0.69	5.23 ± 0.79*

* indicates significantly different from pre-supplementation
value ($P<0.01$; Student's paired t-test)

The distribution of genotypes within the study population and
relationship with TNF-α production and the influence of fish
oil on TNF-α production are discussed above.

2.   Influence of TNF-α, LT-α and IL-6 genotype on response to fish oil

The suppressive effect of fish oil among high TNF-α producers occurred irrespective of TNF-α, LT-α or IL-6 genotype (Table 6). However, there was a significant interaction between TNF-α genotype and inherent TNF-α production in determining the extent of the decline in TNF-α production which followed fish oil supplementation (*P* for interaction = 0.035; two-factor ANOVA).

Table 6

		TNF-α genotype		LT-α genotype		
		1/1	1/2	B1/B1	B1/B2	B2/B2
Tertile of inherent TNF-α production						
Lowest	Before FO	1479 ± 602	1294 ± 713	1132 ± 556	1562 ± 592	1187 ± 757
	Change	2483 ± 2543[a]	2365 ± 3040[a]	2704 ± 1345	2088 ± 2972	3442 ± 2602
Middle	Before FO	3655 ± 962	3883 ± 953	3910 ± 1066	3544 ± 964	3884 ± 790
	Change	658 ± 3066[b]	1238 ± 3365[a]	2040 ± 2558	203 ± 3398	1049 ± 2680
Highest	Before FO	8653 ± 3126	10748 ± 6127	11570 ± 4391	7553 ± 1791	10464 ± 5507
	Change	-2923 ± 4429[c]	-6388 ± 8297*[b]	-7161 ± 7426	-3475 ± 2954	-3246 ± 7800

Further analysis showed that the decline in TNF-α production among individuals in the highest tertile of pre-supplementation TNF-α production was significantly greater (*P* = 0.02) if they possessed the TNF1/2 genotype than if they possessed the TNF1/1 genotype (Table 6). The interaction between LT-α genotype and inherent TNF-α production in determining the extent of the decline in TNF-α production which followed fish oil supplementation failed to reach statistical significance (*P* for interaction = 0.062; two-factor ANOVA). Fish oil was able to suppress production of TNF-α by cells from some individuals among the low and medium tertiles of inherent TNF-α production. The TNFB1/B2 allele appeared to be important in determining sensitivity to fish

oil among these individuals. Thus, all 8 subjects in the
lowest tertile of inherent TNF-α production who responded to
fish oil treatment with a reduction in TNF-α production had
the TNFB1/B2 genotype. In the middle tertile of inherent TNF-
5   α production, 12 out of 16 subjects who responded in this way
had the TNFB1/B2 genotype. In the highest tertile of inherent
TNF-α production, the TNFB1/B2 genotype only characterised
half of the subjects (16 out of 32) who responded to fish oil
with a reduction in TNF-α production.
10

Discussion

Our data suggest that the sensitivity of an individual to the
suppressive effects of n-3 PUFAs on TNF-α production is linked
15 to the inherent level of production of the cytokine by cells
from the individual prior to supplementation and to genetic
variation encoded by, or associated with, the TNF-α -308, LT-α
+252 and IL-6 -174 SNPs. Paradoxically fish oil appears to
enhance TNF-α production in some subjects, particularly those
20 in the lowest tertile of pre-supplementation production. The
ability of fish oil to enhance rather than reduce TNF-α
production is not unexpected. During inflammation
phospholipase A_2 hydrolyses membrane phospholipids, thus making
arachidonic acid available for the production of the pro-
25 inflammatory eicosanoids prostaglandin E_2 (PGE_2) and
leukotriene B_4 (LTB_4). In vitro studies have shown that PGE_2
and LTB_4 have opposing effects on pro-inflammatory cytokine
production, the former having an inhibitory and the latter a
stimulatory influence (Endres et al., N Eng J Med **320**:265-71
30 (1989) and Choi et al., Cell Immunol **170**:178-84 (1996)). Fish
oil may alter pro-arachidonic acid in the cell membrane. Such
an effect would decrease PGE_2 and LTB_4 production and increase
the formation of PGE_3 and LTB_5. These eicosanoids have lower

bioactivity than PGE_2 and LTB_4. Thus, the overall effect on
TNF-α production (inhibition or stimulation) will depend upon
the balance among the different stimulatory and inhibitory
eicosanoids produced from arachidonic acid and
5   eicosapentaenoic acid.

As outlined earlier, genetic influences are important in
influencing TNF-α production. The frequencies of the TNF1,
TNF2, TNFB1, TNFB2, IL-6GG and IL-6CC alleles in the present
10 study accord closely with published values from studies of
healthy British and other European subjects (Perrey et al.,
Transpl Immunol **6**:193-7 (1998), Fanning et al., Tissue
Antigens **50**:23-31 (1997) and Brinkman et al., Br J Rheumatol
36:516-21 (1997)) and are in close agreement with those
15 derived from independent studies in our laboratory (Howell et
al., Eur J Immunogenet **29**:17-23 (2002)). Thus, the group of
subjects studied here is representative of the population from
which it is drawn, at least with respect to the frequencies of
TNF-α, LT-α and IL-6 genotypes examined. The observed
20 positive association between TNFB2 homozygosity and inherent
TNF-α production confirms the findings of Stuber et al., Crit
Care Med **24**:381-4 (1996) and Pociot et al., Eur J Immunol
23:224-31 (1993). However, we do not confirm an association
between TNF-α -308 genotype and TNF-α production. When the
25 genetic characteristics of individuals in the three tertiles
of inherent TNF-α production are examined in relation to the
ability of fish oil to reduce TNF-α production, a complex
interaction was apparent. The results of the present
investigation suggest firstly that most (in this case 86%)
30 individuals with a high inherent level of TNF-α production are
sensitive to the anti-inflammatory effects of fish oil,
secondly that medium and high inherent TNF-α production is
associated with homozygosity for the TFNB2 allele, thirdly

that individuals with medium or low levels of inherent TNF-α
production are more likely to experience the anti-inflammatory
effects of fish oil if they are heterozygous for the TNFB
alleles and fourthly that possession of the IL-6 -174 CC

5   genotype is associated with a lower level of responsiveness to
the anti-inflammatory effects (with regard to TNF production)
of fish oil. It is also the case that individuals who are
TNFB2/B2 are less likely to experience the anti-inflammatory
effects of fish oil, independent of their level of inherent

10   TNF-α production.

The present study is the largest investigation into the
effects of dietary fish oil supplementation on ex vivo TNF-α
production from human PBMC currently reported in the

15   scientific literature (Calder et al., Nutr Res **21**:309-41
(2001)). The data from this study when aggregated without
consideration of each subjects inherent ex vivo TNF-α
production, or TNF-α, LT-α or IL-6 genotype, agree with other
studies which suggest that fish oil does not exert a

20   modulatory effect on such production (Schmidt et al., Scand J
Clin Lab Med **56**:87-92 (1996); Cooper at al., Clin Nutr **12**:321-
8 (1993); Blok et al., Eur J Clin Invest **27**:1003-8 (1997);
Molvig et al., Scand J Immunol **34**:399-410 (1991) and Yaqoob et
al., Eur J Clin Nutr **30**:399-410 (2000)). A wide range of

25   doses of fish oil have been employed in similar studies (0.55
to 6 g n-3 PUFAs/d). Suppressive effects of fish oil on TNF-α
production have generally been demonstrated in studies that
have employed doses of n-3 PUFAs greater than that employed in
the present study (Endres et al., N Engl J Med **320**:265-71

30   (1989); Gallai et al., J Neuroimmunol **56**:143-53 (1995) and
Kelley et al., Lipids **34**:317-24 (1999)). This is not,
however, universally the case as some studies using higher
doses have shown no effect on TNF-α production (Blok et al.,

Eur J Clin Invest **27**:1003-8 (1997); Molvig et al., Scand J Immunol **34**:399-410 (1991) and Yaqoob et al., Eur J Clin Nutr **30**:399-410 (2000)). Of the five studies (Schmidt et al., Scand J Clin Lab Med **56**:87-92 (1996); Cooper at al., Clin Nutr

5 **12**:321-8 (1993); Blok et al., Eur J Clin Invest **27**:1003-8 (1997); Meydani et al., J Clin Invest **92**:105-13 (1993) and Molvig et al., Scand J Immunol **34**:399-410 (1991)) only that by Meydani et al demonstrated an inhibitory effect of fish oil on TNF-α production. However, in this latter study fish oil was

10 given to subjects consuming a low fat diet. In this dietary situation competition between n-6 PUFAs from the diet and n-3 PUFAs from the supplement for incorporation into cell structure would have been less than in the present study.

15 The results of the present study, taken with those of other studies performed on the effects of fish oil supplements on TNF-α production, indicate that the interaction of n-3 PUFA intake and cytokine biology is complex. While the dose of fish oil that is given may be a determinant of whether a

20 suppressive effect of the oil on TNF-α production can be demonstrated at a whole population level, our data suggest that differing sensitivities of individuals to the effects of fish oil, due to genetic variation encoded by, or associated with, the TNF-α -308, LT-α +252 and IL-6 -174 SNPs, may limit

25 the effectiveness of moderate doses of fish oil as an anti-inflammatory agent. The greater understanding of the precise nature of the determinants of sensitivity to fish oil provided by the present application will enable fish oil supplementation to be used for influencing inflammation with

30 greater precision than is presently the case.